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Immune responses limit adenovirally mediated gene expression in the adult mouse eye

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In order to investigate the immunological consequences of gene transfer to the eye using viral vectors, adenovirus carrying a lacZ reporter gene (AV.LacZ) was injected either subretinally, subconjunctivally or into the anterior chamber of three groups of adult mice: immunocompetent or transiently immunosuppressed BALB/c mice and congenic immunodeficient nude mice. Adenovirally mediated lacZ expression persisted for approximately 3 weeks following injection of the vector into the anterior chamber, retina or extra ocular tissues of the conjuctiva of BALB/c mice. It appears that T cell-mediated immune responses limit the duration of AV-mediated ocular gene expression in adult mice since lacZ gene expression was detected for at least 15 weeks in T cell-deficient BALB/c nude mice, although the level of transgene expression decreased with time. Since intra-ocular AV-mediated gene expression was not significantly longer than extra-ocular expression, it appears that the eye is not normally immune-privileged with respect to viral vectors. Inflammatory cells were detected in the vitreous after anterior chamber injection and in the retina after subretinal injection of adenovirus. The presence of both CD4+ and CD8+ T cells was established by immunophenotyping. Reinjection of BALB/c mice resulted in rapid decline in reporter gene expression, but successful readministration was possible in the case of immunodeficient nude mice. However, after transient depletion of T cells, achieved by intraperitoneal injection of both CD8- and CD4-specific antibodies, the duration of expression in BALB/c mice was longer in the eye (at least 12 weeks, again with decrease in level over time), than in extra-ocular tissues (8 weeks) provided the animal was not reinjected with virus, raising the possibility of partial ocular immune-privilege after transient immunosuppression.

Keywords: *adenovirus; immune response; immune privilege; eye*

Introduction

The eye has a number of advantages as a target organ for gene delivery. It is easily accessible and the tissues may be examined *in vivo* by ophthalmoscopy. In addition there are blood–retinal and blood–aqueous barriers which may concentrate vectors in the target area. The eye may be used for testing gene delivery to a wide range of tissues since it contains: endothelium (cornea), epithelium (cornea, ciliary body, iris), muscle (ciliary body) and neurons (retina). It may also serve as a valuable model system to test gene therapy strategies for the brain, whose neurons are more difficult to target than those in the neuroretina. A number of vector systems have been evaluated in the eye, including adeno-associated virus,[1–4] herpes simplex virus[5] and liposomes.[6] However, the most widely used *in vivo* system is that of adenovirus (AV) mediated gene transfer.[7–16] Anterior chamber injection of the virus in adult mice results in efficient transduction of corneal endothelium, iris pigment epithelium and trabecular meshwork, whereas subretinal injection results in efficient transduction of retinal pigment epithelium (RPE) cells with very few photoreceptor cells (reviewed by Ali *et al*[17]). However, host immune responses to vector and transgene currently present a general problem for long-term gene delivery. There has been particular concern about the imunogenicity of AV vectors. The current generation of AV vectors have deletions in their E1 and E3 regions, but are nevertheless able to produce viral proteins. Immune responses against vector-encoded proteins have been demonstrated after *in vivo* transduction of a variety of tissues in humans and mice. In the mouse this has been demonstrated in the heart and lung[18] muscle[19,20] and liver.[21] Neutralising antibodies to adenoviral capsid proteins prevent effective re-administration of the vector, and cytotoxic T lymphocytes (CTL) mediate the destruction of transduced cells, thus reducing the level of transgene expression over time.[22–24] Immune responses to the transgene have also been demonstrated, although it is possible that there is some vector-dependent or tissue-dependent variation in the immunogenicity of the transgene.[20] The eye however, like the rest of the central nervous system, is an immune-privileged site.[25] Allogeneic retinal grafts implanted in the anterior chamber and vitreous cavity survive significantly longer than transplants to other nonimmune privileged sites, such as the subconjunctival space. It is still not clear to what extent, if any, immune privilege extends to the subretinal space.[26,27] We wished to determine whether the eye is also immune-privileged with respect to viral vectors and what practical consequences any privilege might have with respect to

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gene therapy for ocular disorders. We therefore carried out a comparison of the duration of AV-mediated gene expression following injection of adenovirus intra- and extra-ocularly in immunodeficient and immunocompetent animals.

Results

The duration of AV-mediated *lacZ* expression following intra-ocular and extra-ocular (subconjunctival) injection was determined in both immunocompetent and immunodeficient mice. Figure 1 provides a schematic of the injection routes used. Animals were killed at periods ranging from 1 day to several months after injection and the eyes and extra-ocular tissues incubated overnight with X-gal to demonstrate β-galactosidase (β-gal) activity. All were examined under a dissecting microscope and some were also embedded in paraffin wax and sectioned to determine the specificity of tissue expression. Anterior chamber (AC) injections resulted mainly in the transduction of corneal endothelium and iris pigment epithelium, subretinal (SR) injections resulted in the transduction of RPE and the occasional neuroretinal cell, whilst subconjunctival (SC) injections resulted mainly in the transduction of conjunctival fibroblasts (Figure 2). Following either AC, SR or SC injection of immunocompetent BALB/c mice, reporter gene expression was observed for the same duration – approximately 3 weeks (Figure 3). However, similar SR injection of CD1 and outbred MF1 mice resulted, on average, in expression for 4 weeks (Figure 3). In order to determine whether an immune response was responsible for the decrease in reporter gene expression in the eye, the vector was injected into immunodeficient BALB/c congenic (*Nu/Nu*) nude mice. These animals have an epithelial cell defect, caused by a mutation in a single gene, which prevents them forming a thymus and as a consequence they lack mature T-lymphocytes. Whilst there was essentially no detectable X-gal staining in any immunocompetent animal by 6 weeks after injection, the nude mice still showed positive cells at least 15 weeks after either AC, SR or SC injection (Figure 3). The level of X-gal staining, however, decreased with time: we only observed 5% of the level of staining in positive eyes 15 weeks after injection compared with that seen in positive eyes 1 week after injection.

Inflammatory cells were observed in some eyes 1 week after intra-ocular injection of AV.LacZ. Inflammatory cells were observed in the vitreous after anterior chamber injection of virus (Figure 4a) and in the retina after subretinal injection (Figure 4b–c). Immunophenotyping revealed the presence of both $CD4^+$ and $CD8^+$ T cells (Figure 4b–c). Having established that cells transduced by AV.LacZ are removed by the host immune response, we examined, using ELISA, the serum levels of anti-AV antibodies in BALB/c mice over time, after SR injection of AV.LacZ. Antibodies were first detected 4 days after injection, the level peaked after 3 weeks and remained more or less constant for several months, although there was some variability in the levels of antibodies detected in different mice (Figure 5).

Serum, taken from animals 1 month after intra-ocular injection with virus, was incubated with virus which was then titrated by plaque assay. The titres were not affected, indicating that at least *in vitro* the antibodies were not neutralising infection. Ten BALB/c animals were subretinally injected with the virus and were then re-injected 1 month later. Eyes from five of these mice were examined for β-gal activity the following day and were all positive, confirming that circulating antibodies were not neutralising infection *in vivo* either. The remaining animals were examined 6 days after the second injection. None of the eyes showed any blue staining, indicative of an adaptive secondary immune response to proteins encoded by virus or transgene. Using ELISA, similar levels of AV- and β-gal-specific antibodies in the serum of immunocompetent BALB/c animals were detected after AC, SR and SC injection of AV.LacZ (Figure 6). The circulating level of AV- and β-gal-specific antibodies in CD1 mice following SR injection of AV.LacZ was similar to

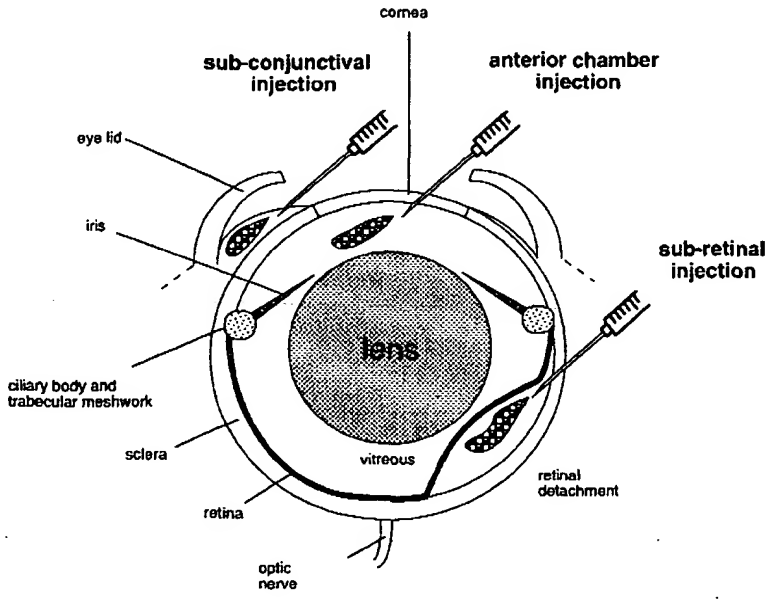

Figure 1 Schematic diagram of a mouse eye. The route of anterior chamber, subretinal and subconjunctival injections is shown.

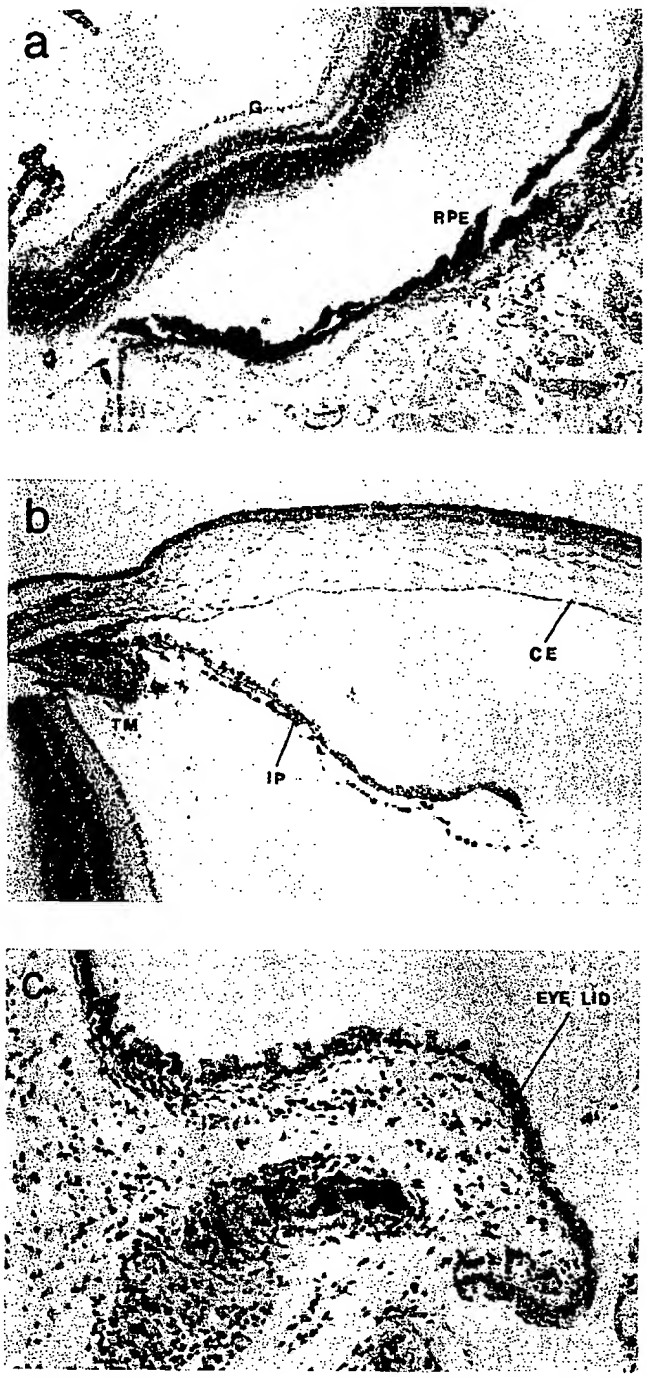

Figure 2 Histology of retina, cornea and conjunctiva following subretinal, anterior chamber or subconjunctival injection of AV.LacZ into BALB/c mice. (a) Blue X-gal staining 2 weeks after subretinal injection represents transduction of retinal pigment epithelium (RPE), the neuroretina consisting of ganglion (G), bipolar (B) and photoreceptor (P) cell layers is untransduced (×10 objective). (b) Transduction of corneal endothelium (CE), iris pigment epithelium (IP) and trabecular meshwork (TM) following anterior chamber injection (×10 objective). (c) Transduction of conjunctival fibroblasts following subconjunctival injection (×20 objective). The 5–10 μm paraffin sections were counterstained with nuclear fast red.

the levels of antibodies detected in BALB/c mice. However, the levels of AV- and β-gal-specific antibodies in the serum of nude mice following SR injection of AV.LacZ was significantly lower.

Whilst nude mice are a useful tool for examining the potential of vectors for therapy when the immunogenicity of the vector may present a problem, an alternative approach is to deplete T cells in normal animals by intraperitoneal injection of CD8- and CD4-specific antibodies. This procedure results in a temporary depletion of T cells, which reappear within 4 weeks following a single injection and thus only induces a transient immune suppression.[28] Depletion of just one T cell subset (either $CD8^+$ or $CD4^+$) by administration of only one of these antibodies did not significantly extend the duration of AV-mediated reporter gene expression (Figure 7). This was despite the fact that CD4 mAb-, but not CD8 mAB-treated animals lacked anti-β-galactosidase and anti-AV antibodies (data not shown). However, administration of a combination of CD4- and CD8-specific antibodies at the time of S.C. injection, allowed reporter gene expression for up to 8 weeks after injection (Figure 8) – an increase in the duration of expression in the extra-ocular tissues by up to 4 weeks. However, after transient immune suppression and SR injection, reporter gene expression lasted up to 12 weeks (Figure 8), an increase of up to 8 weeks. This is despite the fact that the mice had a nearly normal complement of lymphocytes at this time and earlier time-points when examined by flow cytometry.[28] In order to determine whether the difference in duration of gene expression after intra-ocular and extra-ocular injection was due to immunological isolation of the eye established during transient immune suppression, a number of BALB/c mice were transiently suppressed with antibodies and were subjected to monocular SR injections of vector. After 4 weeks, in some of the mice, procedured eyes were subretinally re-injected with PBS to disrupt the blood–retinal barrier, whilst in other animals the contra-

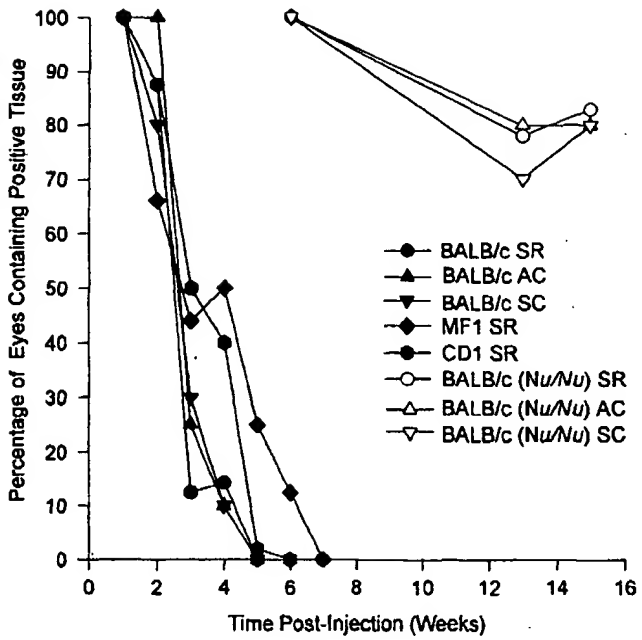

Figure 3 Duration of AV-mediated lacZ gene expression in different mouse strains following various administration routes. The percentage of eyes containing any X-gal-positive tissue following subretinal (SR), anterior chamber (AC) or subconjunctival (SC) injection of AV.LacZ in BALB/c, MF1, CD1 and BALB/c Nu/Nu mice. After incubation, all tissues were carefully examined to detect blue X-gal staining using a dissecting microscope. At each time-point, material from between four and eight mice was examined; in total 200 animals (400 eyes) were procedured.

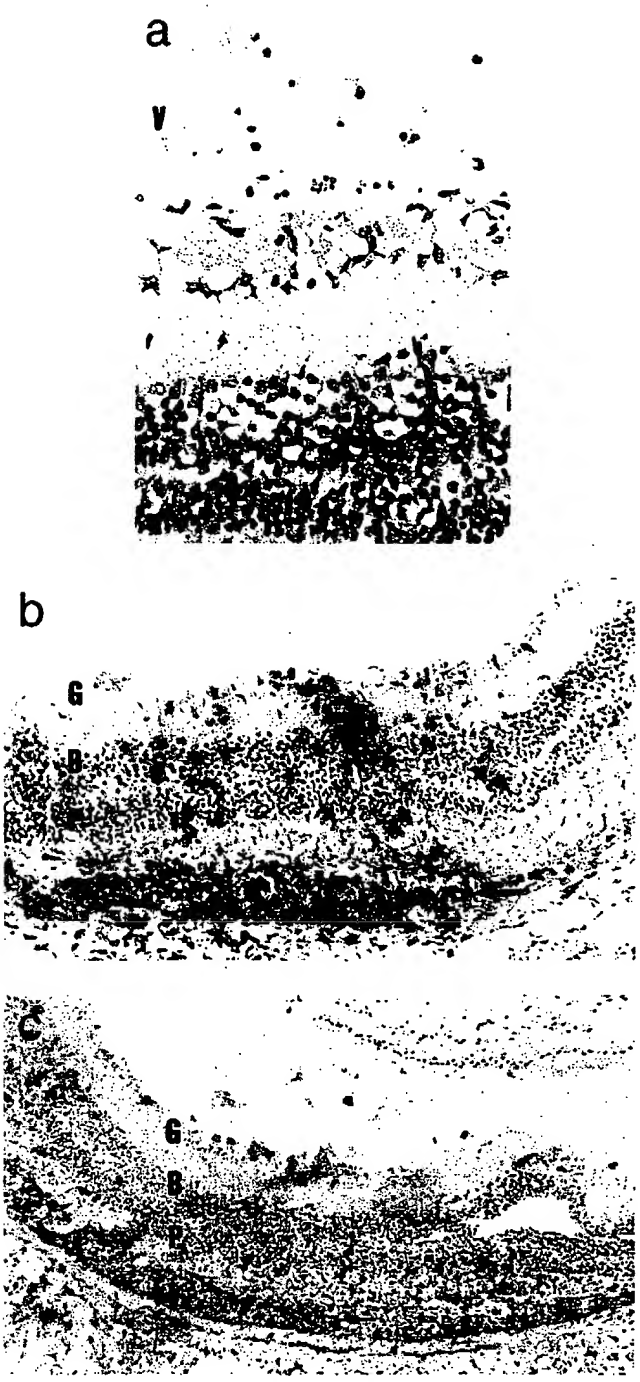

Figure 4 Inflammatory cells may be observed in the eye after intra-ocular injection of Av.LacZ. (a) Six days after anterior chamber injection, inflammatory cells are present in the vitreous (V): 5 μm paraffin section counterstained with H & E (×40 objective): (b) Six days after subretinal injection, CD4⁺ cells may be found in parts of the retina: 8 μm cryosection stained with CD4-specific antibody and counterstained with eosin (×20 objective). (c) Six days after subretinal injection, CD8⁺ cells may also be found in parts of the retina: 8 μm cryosection stained with CD8-specific antibody and counterstained with haematoxylin (×10 objective). The retina is labelled as follows: ganglion (G), bipolar (B) and photoreceptor (P) cell layers. Arrow indicates subretinal infiltration.

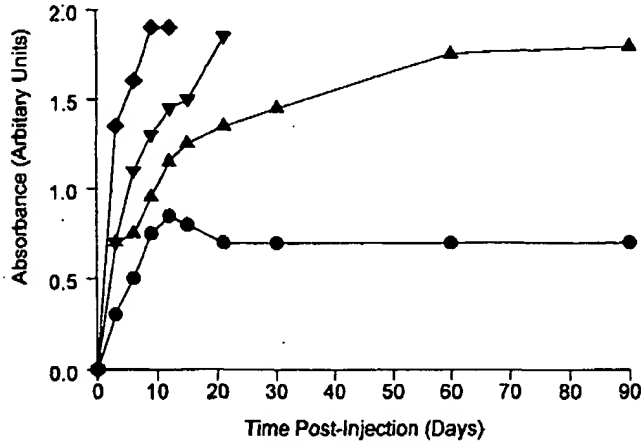

Figure 5 Levels of circulating AV-specific antibodies in mice over time, following subretinal injection of AV.LacZ. BALB/c mice were injected subretinally with 2×10^6 p.f.u. AV.LacZ and bled serially. Plasma samples (1:100 dilutions) from four individual mice were assayed by ELISA to detect AV-specific antibodies. The results represent the optical densities at each time-point.

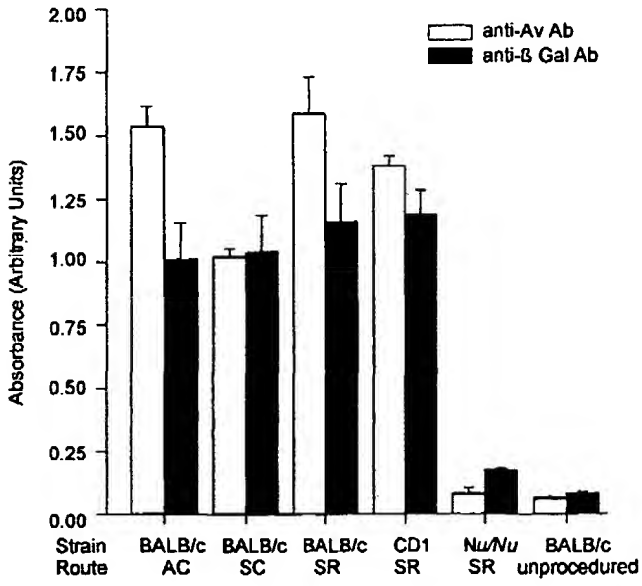

Figure 6 Levels of circulating AV- and β-galactosidase-specific antibodies in different mouse strains following administration of AV.LacZ by subretinal (SR), anterior chamber (AC) or subconjunctival (SC) injection. Plasma was collected from BALB/c, CD1 or BALB/c Nu/Nu mice 3–4 weeks following injection of 2×10^6 p.f.u. AV.LacZ. The samples were diluted 1:100 and assayed to detect AV- or β-gal-specific Ab by ELISA. The results represent the mean ± s.d. optical densities of three animals per group.

lateral unprocedured eye was subretinally injected with the AV.LacZ vector. Injection of PBS into eyes injected 4 weeks previously with AV.LacZ did not significantly reduce the duration of expression compared with immunosuppressed animals which had only received a single SR injection of AV.LacZ (Figures 8 and 9). In contrast, re-exposure of the animal to the virus resulted in rapid loss of transduced cells – within 5 days – in both eyes (Figure 9), indicating immunoactivation. Further evidence for immunoactivation was provided by the circulating antibody response. The level of AV-specific anti-

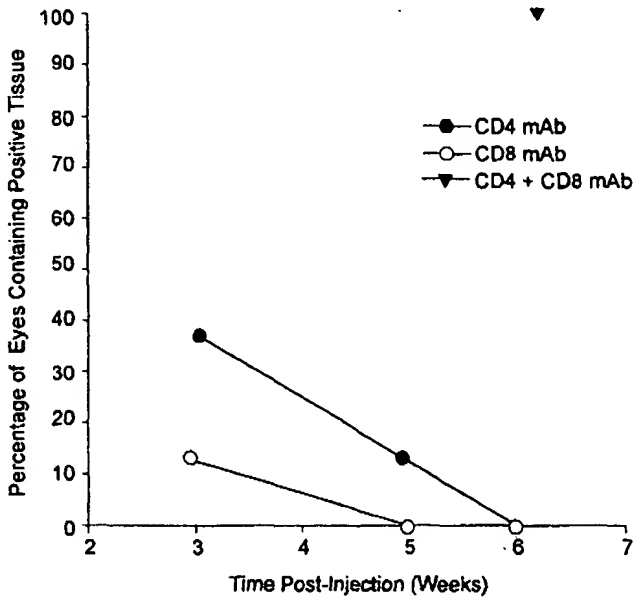

Figure 7 Comparison of the duration of AV-mediated lacZ gene expression in BALB/c mice following subretinal injection after transient depletion of either CD4⁺ or CD8⁺ T cells or transient depletion of both types. The Figure shows the percentage of eyes containing any X-gal positive tissue following injection of 2×10^6 p.f.u. AV.LacZ into mice which have been transiently depleted of T cells by intraperitoneal injection of CD4 (YTS 191) and/or CD8 (YTS 169) specific monoclonal antibodies. Four or five mice were examined at each time-point.

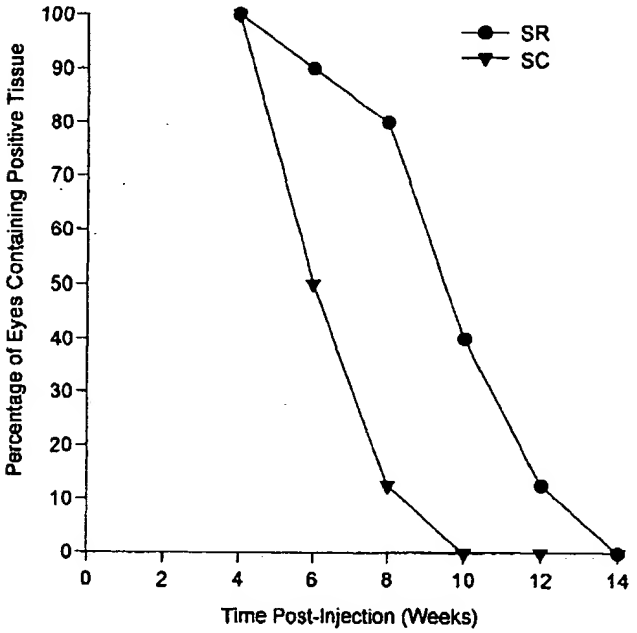

Figure 8 Comparison of the duration of AV-mediated lacZ gene expression in T-cell depleted BALB/c mice following intra-ocular (subretinal) versus extra-ocular (subconjunctival) injection. The Figure shows the percentage of eyes containing any X-gal-positive tissue following injection of 2×10^6 p.f.u. AV.LacZ into mice which have been transiently depleted of T cells by intraperitoneal injection of CD4 (YTS 191) and CD8 (YTS 169) specific monoclonal antibodies. Between six and eight mice were examined at each time-point.

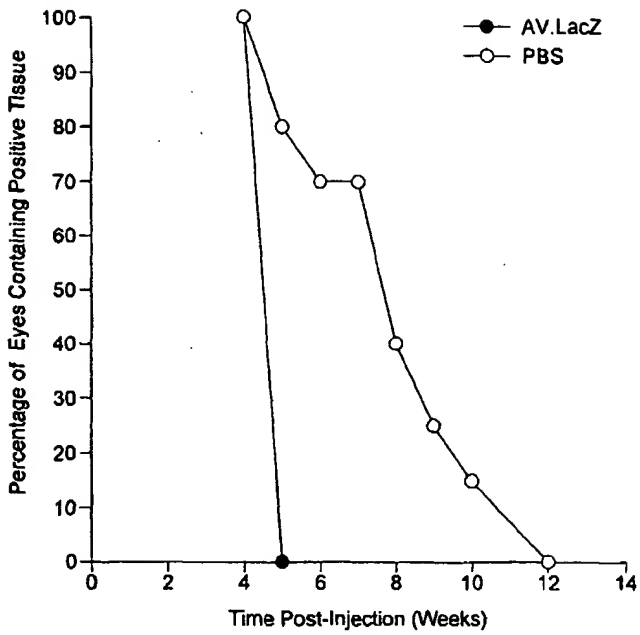

Figure 9 Role of blood–retinal barrier in the immune response to adenoviral vectors. BALB/c mice were transiently depleted of T cells by intraperitoneal injection of 250 μg CD4 (YTS 191) and CD8 (YTS 169) specific monoclonal antibodies and were injected subretinally in one eye with 2×10^6 p.f.u. AV.LacZ. After 4 weeks, the animals were reprocedured. In some mice, procedured eyes were subretinally re-injected with 2 μl PBS to disrupt the blood–retinal barrier, whilst in other animals the contralateral unprocedured eye was subretinally injected with 2×10^6 p.f.u. AV.LacZ in order to prime the immune system. The percentage of eyes containing any X-gal-positive is shown. Between six and eight mice were examined at each time-point.

bodies significantly increased following re-injection of AV.LacZ, but was unaffected by disruption of the blood–retinal barrier alone (Figure 10).

Discussion

Comparisons between reports in the literature of the duration of AV-mediated gene expression in various organs and in the eye are difficult to make because of differences in strains and ages of mice, viral constructs and the amounts of virus injected. However, here we have been able to control for these factors. Our results suggest that there is little difference in the duration of AV-mediated transgene expression between the anterior or posterior segments of the eye and extra-ocular tissues in immunocompetent animals.

Our results demonstrate that the decrease with time in adenovirus-mediated gene expression in the eye is due in large part to an immune response, although it is possible that some of the decrease in expression is due to inactivation of the RSV promoter. It has recently been shown in cultured human corneas in which the endothelium had been transduced by a similar AV vector, that the RSV promoter was down-regulated after 7 days with a concomitant reduction in β-galactosidase activity.[29] Whereas reporter gene expression in BALB/c mice after subretinal or anterior chamber injection of AV.LacZ disappeared by 3 weeks, intra-ocular injection into a strain

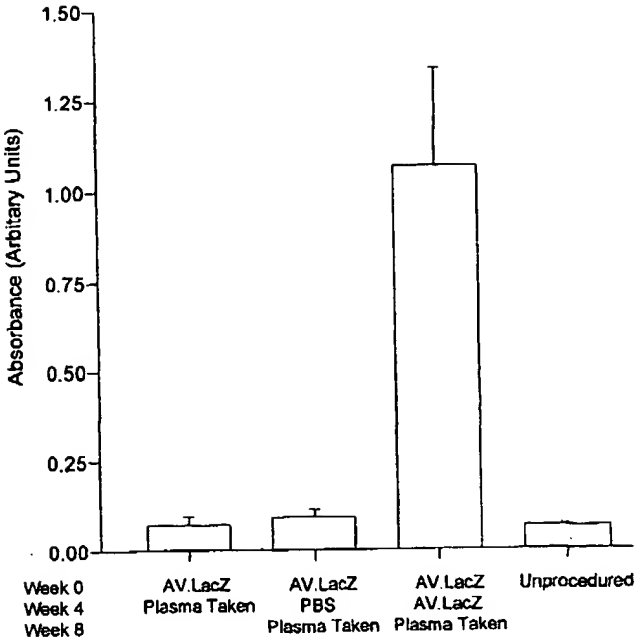

Figure 10 Levels of circulating AV-specific antibodies in transiently immune suppressed BALB/c mice. Subretinal injection of 2×10^6 p.f.u. AV.LacZ in one eye, at the time of administration of 250 μg CD4 (YTS 191) and CD8 (YTS 169) specific monoclonal antibodies, was followed 4 weeks later either by subretinal injection of PBS in the same eye or subretinal injection of 2×10^6 p.f.u. AV.LacZ in the contralateral unprocedured eye. The serum was collected 4 weeks after the procedures. Plasma diluted 1:100 was assayed by ELISA and the results represent mean ± s.d. optical density of three animals per group.

of nude mice, which are congenic to BALB/c, resulted in much longer expression (for at least 15 weeks). Although the immunodeficient nude mice have an epithelial cell defect and some of the tissues transduced by the adenoviral vector are epithelial, it is unlikely that the direct consequence of this defect rather than its effect upon the immune system is responsible for prolonging gene expression. Furthermore, T cell ablation using anti-CD8 and anti-CD4 antibodies also extended the duration of reporter gene expression in the eye. Our results therefore provide strong evidence that the decrease in β-galactosidase expression over time is due to a T cell-mediated immune response. There also appeared to be a strain-dependent variation in the length of expression (the duration of expression was greater in CD1 and MF1 than in BALB/c mice) which is similar to that reported for AV-mediated gene delivery to the liver.[21] This was presumably due to differences in the immune response between different strains of mice. The decrease with time in the level of staining in the eyes of nude mice and transiently suppressed mice may have reflected down-regulation of the RSV promoter or loss of the adenoviral episome. However, since we detected low levels of AV- and β-gal-specific (and presumably T-cell independent) antibodies in these immune-deficient animals following AV.LacZ administration it is also possible that this was due to a slow immune response to transduced cells. Using ELISA we have shown that AV.LacZ-injected immunocompetent mice have circulating antibodies to adenoviral proteins and to β-galactosidase. We observed *lacZ* expression 1 day after re-injecting the mice with the adenoviral vector. Thus the circulating antibodies to the virus were not necessarily neutralising infection *in vivo*. However, $CD4^+$ T-cell dependent antibody responses may have some function in limiting AV-mediated transgene expression in transduced cells. Here we have shown that depletion of T cells with monoclonal antibodies inhibit antibody production and extend the duration of AV-mediated *lacZ* gene expression in the eye. Although immunological tolerance to proteins administered under the cover of transient T cell depletion has been reported,[30] this is unlikely to account for the increased duration of reporter gene expression in this study since the transiently immune suppressed mice were not tolerant to adenoviral proteins or to β-galactosidase following re-administration of AV.LacZ. Our results are consistent with several other studies which have demonstrated that both CD4 and CD8 T cell subsets are important in other organs for eliminating cells transduced with AV.[24]

It is clear that in most *in vivo* systems, the current adenoviral vectors are able to facilitate only short-term gene expression since they elicit strong immune responses. Although the eye is an immune-privileged site with respect to transplanted cells, we have shown here that under normal circumstances this does not necessarily apply to cells in the eye transduced *in vivo* by direct adenoviral vector delivery. However, we have also demonstrated that some degree of ocular immune privilege may be re-established if the vectors are injected intra-ocularly during transient immune suppression. The duration of AV-mediated gene expression under these circumstances was greater when virus was injected intra-ocularly compared with extra-ocularly. One explanation for this privilege might be that transient immune suppression allowed blood–ocular barriers, broken by the injection procedures, to be re-established. In order to investigate this possibility, T cells were transiently depleted in BALB/c mice and the animals subjected to a monocular injection of AV.LacZ. After 4 weeks, at a time when the T cells had returned, the procedured eye was re-injected with PBS. However, AV-mediated transgene expression was not significantly reduced. In contrast, when the contralateral unprocedured eye was injected with AV.LacZ there was a rapid decrease in *lacZ* gene expression. Thus the relative ocular immune privilege established by transient T cell depletion was not solely due to re-establishment of a broken blood–retina barrier preventing access to target antigens. Any potential immune privilege was removed by peripheral priming of the immune system. Following re-administration of adenovirus, transduced cells were rapidly eliminated. Once the immune system becomes primed, any immune-privileged site, including those in the CNS, is lost because primed T cells are able to circulate through these tissues.[31,32] Immunosuppression with monoclonal antibodies in this study prevented the generation of a productive immune response and resulted in prolonged transgene expression. The treatment was unlikely to have prevented all priming events since after re-exposure to AV.LacZ, there is loss of transduced cells within 1 week. Since this process takes approximately 4 weeks in naive animals, the data suggest that priming, as a result of

AV.LacZ exposure during the injection procedure, is most critical in determining the duration of transgene expression. It seems likely that some adenovirus leaks out of the eye following intra-ocular injection. This may occur either by standard drainage from the anterior chamber, or through disrupted barriers. Leakage may result in priming of the immune system and loss of immune privilege in the eye. Immunosuppression prevents a productive immune response on initial vector administration and may therefore allow blood–retinal barriers to reform and transduced cells within the eye to escape normal immune surveillance. Therefore extended gene expression in the eye may, in part, be related to establishment of immune privilege.

We have found no evidence to support an earlier report which suggested that the retina is normally totally immune-privileged with respect to adenoviral vectors.[33] These results are not consistent with our data or that of Li *et al*[14] who also observed a decrease in reporter gene expression with time after subretinal injection of AV.LacZ, this time in neonatal mice. Bennet *et al*[33] compared the level of circulating AV-specific antibodies after subretinal and subcutaneous injection by Western blotting. They were unable to detect any antibodies after intra-ocular injection of virus, whereas a significant level of anti-AV antibodies was detected after subcutaneous injection. Our data, which include the same mouse strain, demonstrate that there can be an immune response to AV which includes circulating antibodies following intra-ocular injection. Using ELISA we could not detect a difference in the levels of circulating anti-AV antibodies after subcutaneous compared with subretinal injection of adenovirus (data not shown). It is therefore difficult to reconcile the results presented here and previously,[33] although it may be due to minor procedural differences preventing priming. However, these authors have noted that AV-mediated *lac*Z expression in the anterior chamber decreased with time[12] and more recently have also reported immunogenicity to adenovirus after intravitreal injection.[34] Borras *et al*[10] have also noted an inflammatory response in rabbits following anterior chamber injection of AV.LacZ.

Our results have a number of implications for the development of ocular gene therapy. First, the eye should not normally be regarded as immune-privileged with respect to viral vectors following direct delivery. This is an important point when considering therapies involving gene delivery to photoreceptor cells. Since they are non-dividing, once eliminated by CTL they would not be replaced. There is some evidence that the immune response to AV vectors may be avoided if vectors are introduced into neonatal animals which have an immature immune system. However, this strategy is unlikely to be of clinical relevance. It appears that repeated administration of adenovirus is not an effective strategy for prolonging ocular gene expression beyond a few days. The immune response to transduced cells may be less of a problem for gene therapy with the use of new less immunogenic viral vectors such as E2a-deleted AV and AAV or nonviral delivery systems. However, it is likely to remain a problem for many gene therapy strategies since the transgene itself may encode an antigenic protein.

Finally, transient immune suppression is an effective way of enhancing gene delivery, particularly to the eye because it may allow some degree of ocular immune privilege to be established, but this does not allow repeated administration of the vector without further suppression.

Materials and methods

Preparation of virus

The AV.LacZ used was an E1-deleted recombinant human 5a adenoviral vector, pXCXRBb, containing a *LacZ* reporter gene driven by a Rous sarcoma virus LTR promoter. This was a kind gift from Dr A Byrnes, Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Public Health, and has been previously described.[35] It was propagated in 293 cells, purified by two CsCl gradients and assayed for infectivity by plaque assay using a conventional protocol.[36] All aliquots of injected virus were from the same CsCl preparation and at a concentration of 10^9 p.f.u./ml.

Animals

Three strains of mice were used HsdOla:MF1; BALB/cOlaHsd; BALB/cOlaHsd-*Nu/Nu* (Harlan Olac, Bicester, UK). All the mice were 8 weeks old at the time of injection (+/− 1 week). Transient T cell ablation was achieved by a single intraperitoneal (i.p.) injection of 250 μg of either or both CD8-specific (YTS 169) and CD4-specific (YTS 191) monoclonal antibodies in 200 μl as described previously.[28]

Injection of virus

Mice were anaesthetized by intraperitoneal injection of 0.2 ml Hypnorm (Janssen Pharmaceutical, Oxford, UK), and Hypnovel (Roche, Welwyn Garden City, UK) mixed 1:1:6 with distilled water. For subretinal injections, the pupils were dilated with 1% Tropicamide (1% Mydriacyl; Alcon Laboratories, Watford, UK) and the eyes were protruded by gentle pressure on the animal's mandible. Once the eye had been proptosed, it was held in this position by a rubber sleeve which was placed around the eye with a pair of forceps. The procedure is best described by analogy with a button hole, in which the rubber sleeve is the button hole and the mouse eye the button. It was held in position by the sleeve which has a slit in the centre to fit the eye. The pressure of the rubber sleeve on the eye was always moderate and did not block the circulation. Using this technique made fixation with ocular muscle sutures unnecessary. Subsequently the eye was covered with 2% hypromellose (methylcellulose) in saline and a small cover slip fitted. This allowed surgery to be performed under direct retinoscopy through an operating microscope. The tip of a 1.5-cm, 34-gauge hypodermic needle (Hamilton, Switzerland) was guided in between the coverslip and the rubber sleeve to the sclera of the mouse eye and then injected tangentially through it causing a self-sealing wound tunnel. The needle tip was brought into focus between the retina and retinal pigment epithelium and approximately 2 μl of PBS or viral suspension (containing 2×10^6 p.f.u. of AV.LacZ) was injected to produce a retinal detachment.

For injection of 2 μl of viral suspension into the anterior chamber, the eye was merely protruded using the rubber sleeve and additionally stabilised by holding an extra-ocular muscle with a pair of fine forceps. The

needle was tangentially inserted into the anterior chamber, tunnelling through the cornea in parallel with, and in close proximity to, the limbus. For subconjunctival injections, the eye was stabilised by holding an extraocular muscle with forceps and the needle was inserted through the conjunctiva close to the limbus. Two microlitres of viral suspension was injected producing a prominent subconjunctival bleb.

X-gal reaction

At various time-points following SR or AC injection of virus, animals were killed by cervical dislocation and the enucleated eyes were prefixed by immersion in 10% neutral buffered formalin for 45 min. The cornea and lens were removed and the eye cups rinsed in PBS and incubated overnight at room temperature with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Calbiochem, La Jolla, CA, USA) in a solution containing 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, in PBS. After SC injection, the eye was removed with eyelids and conjunctiva, prefixed and incubated in X-gal as above.

Histology and immunohistochemistry

After incubation with X-gal, the tissue was fixed in 10% neutral buffered formalin, embedded in paraffin wax and sectioned at a thickness of 5–10 μm. Sections were counterstained with nuclear fast red and examined by light microscopy. Eyes which were used for immunohistochemistry were snap frozen in liquid nitrogen, and embedded in OCT. Cryostat sections of 8 μm were cut and fixed in acetone for 10 min at room temperature. The sections were then incubated with rat mAb specific for CD4 and CD8 and the antibodies detected by indirect immunoperoxidase staining as previously described.[37] Sections were counterstained with haematoxylin.

Detection of antibodies to adenovirus or β-galactosidase

Blood was taken from the tail vein and plasma separated after a brief centrifugation. These were stored at −20°C before assay. Ninety-six well MaxiSorp immunoplates (Nunc, Roskilde, Denmark) were coated with either 1×10^6 p.f.u. pXCXRBb or 1 μg β-galactosidase protein (Sigma, Poole, UK) in 50 μl PBS overnight at 4°C. The wells were rinsed with three washes of PBS containing 0.05% Tween-20, and protein binding sites blocked by a 1-h incubation of 50 μl of PBS containing 10% foetal calf serum (FCS). Different dilutions of mouse plasma, typically 1:100, in 50 μl PBS containing 10% FCS were added to the wells. The plates were incubated for 2 h at room temperature (RT). The wells were rinsed three times as before. Peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, High Wycombe, UK), diluted 1:2000 in PBS/10% FCS, were then added for 1 h at RT. The wells were rinsed six times as before and incubated with chromagen substrate (100 μM OPD (*o*-phenylenediamine)-free base (Sigma, Poole, UK) in PBS containing 0.3% H_2O_2 for 30 min at RT). The reaction was stopped with 3 N HCl and optical densities measured at 405 nm. Data from the linear part of the titration curve was plotted.

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